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Selection of Genetic Variants of Simian Immunodeficiency Virus in Persistently Infected Rhesus Monkeys

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Genetic and antigenic variation may be one means by which lentiviruses that cause AIDS avoid elimination by host immune responses. Genetic variation in the envelope gene (*env*) was studied by comparing the nucleotide sequences of 27 clones obtained from two rhesus monkeys infected with molecularly cloned simian immunodeficiency virus. All 27 clones differed from each other and differed from the input clone in the gp120 (*SU*) portion of the envelope gene. Nucleotide substitutions were shown to accumulate with time at an average rate of 8.5 per 1,000 per year in *SU*. Surprisingly, the majority of nucleotide substitutions (81%) resulted in amino acid changes. Variation in *SU* was not random but occurred predominantly in five discrete regions. Within these variable regions, a remarkable 98% of the nucleotide substitutions changed the amino acid. These results demonstrate that extensive sequence variability accumulates in vivo after infection with molecularly cloned virus and that selection occurs in vivo for changes in distinct variable regions of *env*.

Simian immunodeficiency virus (SIV) is a member of the lentivirus subfamily of retroviruses. This group also includes human immunodeficiency virus (HIV), equine infectious anemia virus, visna virus, and caprine arthritis-encephalitis virus. Members of the lentivirus subfamily establish long-term, persistent infections resulting in chronic, nononcogenic, debilitating disease.

Antigenic variation during persistent infection has been documented for equine infectious anemia virus (27, 35), visna virus (2, 38), and caprine arthritis-encephalitis virus (7). Equine infectious anemia, in particular, is characterized by recurrent clinical episodes of fever, hemolytic anemia, bone marrow depression, lymphoproliferation, immune complex glomerulonephritis, and persistent viremia (27). Virus neutralization assays have shown that plasma recovered from an infected animal can effectively neutralize virus isolated from earlier febrile episodes but cannot neutralize virus isolates recovered during subsequent febrile episodes (27, 35). Antigenic variation is thus one mechanism that lentiviruses may use to evade the host immune system. In previous studies of antigenic variation with the ungulate lentiviruses, plaque-purified virus was not always used. Molecularly cloned virus was never used in these previous studies, and the genetic changes contributing to antigenic variation have not been documented. Furthermore, the ungulate lentiviruses are quite distant from HIV, and it is difficult to extrapolate the significance of findings in these systems to AIDS in humans. The SIVs are the closest known relatives of the HIVs, and they share with their human counterparts extensive similarity in genetic and biological properties (for reviews, see references 4 and 8).

Comparison of the nucleotide sequences of SIV, HIV type 1 (HIV-1), and HIV-2 has revealed extensive genomic variability among these primate lentiviruses, particularly in the envelope regions (see sequences in GenBank). Studies with sequential isolates of HIV-1 have demonstrated heterogeneity within virus isolated at any one time and fluctuations among virus populations over time in infected individuals (10, 26, 34, 42). One question which arises from these studies is whether such heterogeneity is generated by rapid evolution of HIV-1 over time in infected individuals or whether such diversity may have been originally present in the infecting virus inoculum. Studies on sequential isolates of HIV-1 are usually complicated further by lack of information on the time of infection in these individuals, making precise measurement of nucleotide substitution rates impossible in this system. Characterization of the precise rate and nature of nucleotide substitutions is best made by using molecularly cloned virus of known sequence in an experimental animal model system in which the exact time of infection is known.

In this report we describe the study of envelope gene variation in rhesus monkeys infected with the molecularly cloned virus SIVmac239. We have documented the extent to which genetic changes accumulate in *env* with time of in vivo infection and have characterized the nature of the nucleotide substitutions. Our results show that molecularly cloned SIV rapidly evolves into a complex mix of genotypes during infection of a single individual. The data further suggest that variation in five discrete segments results from selective forces operating in vivo. The host immune response is likely to be a major source of this selective pressure.

MATERIALS AND METHODS

Virus. The SIVmac239 pathogenic molecular clone and its complete sequence have been described previously (18, 19, 28, 30a).

Cells and cell lines. Macaque peripheral blood lymphocytes were obtained from blood samples collected in preservative-free heparin by banding over sodium diatrizoate-Ficoll (1.077 to 1.080 g/ml at 20°C; Organon Teknika Corp., Durham, N.C.). Peripheral blood lymphocytes were stimulated with 1 μg of phytohemagglutinin per ml for 48 h, washed free of lectin, and incubated in RPMI 1640 with 10% fetal calf serum, interleukin-2 (lectin-free T-cell growth factor; Electro-Nucleonics, Inc., Fairfield, N.J.), penicillin, and streptomycin. Continuously growing human $CD4^+$ cell lines HuT 78 and CEM × 174 (16) were grown in RPMI 1640 medium with 10% fetal calf serum.

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Experimental infection of rhesus monkeys. Two juvenile rhesus macaques (*Macaca mulatta*) previously negative for SIV antibody were selected from the New England Regional Primate Research Center colony. Rhesus macaque number 243-86 (Mm243-86) was inoculated intravenously on 4 August 1987 with 1 ml of virus derived from transfection of molecularly cloned SIVmac239 into HuT 78 cells (28). The virus was grown in HuT 78 cells for 41 days and had 29,592 cpm of reverse transcriptase activity per ml on the day of inoculation. Mm326-87 was inoculated intravenously on 1 June 1989 with 1 ml of virus derived from transfection of molecularly cloned SIVmac239 into rhesus macaque peripheral blood lymphocytes. The virus was grown in macaque peripheral blood lymphocytes for 18 days and had 100,000 cpm of reverse transcriptase activity per ml on the day of inoculation. Cell-free virus stocks for inoculation were prepared by centrifugation of infected cell cultures and filtration of viral supernatants through 0.45-μm- pore-size filters. Heparinized blood samples were collected from the macaques at intervals after the virus inoculation and were used for preparation of total cell DNA, for virus recovery, and for monitoring antibody response.

DNA preparation. Total cell DNA (36) was prepared from peripheral blood mononuclear cells (PBMCs) obtained at 69 and 93 weeks postinfection and from a lymph node biopsy taken at 69 weeks from Mm243-86. A fraction of the lymph node taken at 69 weeks and the PBMCs taken at 93 weeks were each cocultivated with CEM × 174 cells for 51 and 41 days, respectively, and cells infected with recovered virus were used to prepare Hirt supernatant DNA (14). A fraction of a 6-week PBMC sample from Mm243-86 was cocultivated with HuT 78 cells for 68 days, and cells infected with recovered virus were also used to prepare Hirt supernatant DNA. For the control clones, the virus stock used to inoculate Mm243-86 was used to infect HuT 78 cells, and Hirt supernatant DNAs were prepared 31 and 55 days later. From Mm326-87, total cell DNA was prepared at 43 weeks postinfection from a PBMC sample.

PCR amplification. Total cell and Hirt supernatant DNAs were used as templates in a polymerase chain reaction (PCR) to specifically amplify full-length *env* sequences between nucleotides 6544 and 9249 (numbering system of Regier and Desrosiers [30a]). Because the copy number of SIV DNA was much lower in blood and lymph nodes than in cells infected with the recovered virus, it was necessary to perform two rounds of PCR amplification for blood and lymph node samples to obtain sufficient quantities of DNA for cloning (PCR amplification method adapted from reference 39). One round of PCR refers to 30 cycles of amplification. The first round of PCR was performed with an outer set of oligonucleotide primers (nucleotides 6474 through 6493, 5′-AGTGTTGCTACCATTGCCAG-3′; nucleotides 9279 through 9298, 5′-AGCTGGGTTTCTCCATGGAG-3′), whereas the second round was performed with a second set of primers (nucleotides 6544 through 6573, 5′-GAGAAGAAGA GCTCCGAAAAAGGCTAAGGC-3′; nucleotides 9220 through 9249, 5′-TGTCCCTCACAAGAGAGTGAGCTCAA GCCC-3′) complementary to the amplified product of the first round. The second set of primers contained *Sst*I sites (underlined) for cloning; an A→G mutation was made in the primer from nucleotides 6544 through 6573 at position 6554 to create one of the *Sst*I sites. By using two sets of primers, nonspecific amplification was reduced. By using two rounds of PCR, the level of amplification was greatly increased. For the Hirt supernatant DNA, only one set of primers was used (nucleotides 6544 through 6573 and 9220 through 9249) in one round of PCR. One microgram of PBMC or lymph node DNA or an empirically determined quantity of Hirt supernatant DNA was added to a PCR reaction containing the following: 1.5 mM Mg^{2+}, 200 μM each dinucleoside triphosphate, 0.2 μM each oligonucleotide primer, and 2.5 U of Taq polymerase. The total reaction volume of 100 μl was overlaid with light mineral oil. DNA was amplified for 30 cycles with the following cycle profile: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. An auto extension of 10 s was added to each PCR cycle, and a 10-min final extension at 72°C was added to the last cycle. PCR contamination was avoided by taking precautions similar to those outlined by Kwok and Higuchi (20). In addition, negative DNA samples prepared simultaneously with positive DNA samples were amplified in each PCR experiment as controls, along with controls containing only the reagent (no template DNA added).

Molecular cloning. Approximately 5% of each PCR reaction was analyzed on 1.0% agarose gels. The DNA was transferred to nitrocellulose filters (Schleicher & Schuell, Inc.) and hybridized with the gel-purified, ^{32}P-labeled *Sst*I fragment (*env*) of SIVmac239. The remaining 95% of the PCR reaction was precipitated with ethanol and digested with *Sst*I. Gel purification of the *Sst*I fragment was necessary only for the 6-week Hirt supernatant DNA from Mm243-86 due to a higher amount of nonspecific DNA in this sample. After digestion with *Sst*I, the PCR-amplified material was ligated with the *Sst*I-cleaved vector pBS^- (Stratagene). Approximately 25% of each ligation was used to transform *Escherichia coli* XL1-Blue competent bacterial cells (Stratagene). The F′ episome of XL1-Blue contains the *lacI*^{q}ZΔM15 mutation providing α-complementation of the β-galactosidase gene for blue/white color selection. Single white colonies were picked and screened by a colony hybridization method employing the envelope-specific, gel-purified *Sst*I fragment of SIVmac239. Positive colonies were selected for small-scale plasmid preparations, and clones containing the desired 2.7-kbp insert were finally selected for large-scale plasmid preparations and sequence analysis.

DNA sequencing. The double-stranded plasmid clones were sequenced by the primer-directed dideoxy-chain termination method (37) with Sequenase (United States Biochemical Corp.) and a series of internal primers synthesized on a Cyclone DNA synthesizer (Biosearch, Inc.). ^{35}S-labeled sequencing reactions were electrophoresed on 6% polyacrylamide gels with 8 M urea. Sequences were analyzed with IBI-Pustell DNA analysis software.

Nucleotide sequence accession number. The nucleotide sequences (data not shown) of the 27 clones from Mm243-86 and Mm326-87 and the 5 control clones have been filed with GenBank under accession no. M61062 through M61093.

RESULTS

Infection of rhesus monkeys with molecularly cloned virus. Two juvenile rhesus monkeys (Mm243-86 and Mm326-87) were inoculated intravenously with virus derived from transfection of molecularly cloned SIVmac239. The SIVmac239 clone is known to yield virus that causes AIDS in rhesus monkeys (18), and its complete sequence is known (30a). A persistent infection was established in both animals, since SIV was repeatedly isolated from their blood over time. The animals also developed strong stable antibody responses to SIV as measured by enzyme-linked immunosorbent assays, Western immunoblots, and neutralization assays (data not shown). Mm243-86 was euthanatized when death appeared

imminent after persistent infection with SIV for 2.9 years. Mm326-87 is presently asymptomatic 1.2 years after infection.

Using the PCR amplification and cloning strategy described in Materials and Methods, 98 full-length *env* clones were obtained from PBMCs, lymph node tissue, and cells infected with recovered virus at 6, 69, and 93 weeks postinfection from Mm243-86. At the time these clones were obtained, Mm243-86 was asymptomatic. By using the same procedures, 13 full-length *env* clones were obtained from Mm326-87 from a 43-week PBMC sample. The DNA sequences of *SU* of 21 clones from Mm243-86 and 6 clones from Mm326-87 were determined. This represents 42,406 bp of sequence information from these two animals.

Variable regions of the SIV envelope. The translated amino acid sequences of the 22 late-time-point clones from Mm243-86 and Mm326-87 revealed a total of five variable regions in the gp120 region of *env* (Fig. 1 and 2). Variable regions 1 and 2 were located near the amino terminus of gp120 between amino acids 115 and 142 and between amino acids 188 and 202, respectively. Variable region 3 was located between amino acid residues 371 and 376, between the regions corresponding to the variable cysteine loop (40) and the CD4 binding domain (21) of HIV-1. Variable regions 4 and 5 were found immediately flanking the region corresponding to the CD4 binding domain of HIV-1 between amino acid residues 404 and 426 and between residues 473 and 478, respectively.

In addition to the five variable regions in gp120, there was also a variable region in the signal peptide of late-time-point clones from Mm243-86 (Fig. 1). In late-time-point clones from both Mm243-86 and Mm326-87, there was also selection for particular amino acid changes at several points in gp120. There was selection for glutamine at amino acid residue 24, methionine at residue 67, and glycine and asparagine at residue 511 (Fig. 1 and 2).

Variable regions V2, V3, V4, and V5 are located at potential sites of N-linked glycosylation (N-X-T or N-X-S), whereas V1 is immediately adjacent to a potential N-linked glycosylation site. Within variable region 4, an alanine-to-threonine change in 21 of 22 late-time-point clones resulted in the creation of a new potential N-linked glycosylation site (Fig. 1 and 2).

Two of the late-time-point clones (T69 BL 1-21 in Fig. 1 and T43 BL 20-8 in Fig. 2) had deletions in variable region 1. Both deletions were two amino acids in length, did not change the reading frames, and were located in slightly different locations in the two clones. Half of the late-time-point clones from Mm243-86 had deletions in variable region 4, and the deletions were found in clones obtained from PBMCs and lymph nodes as well as recovered virus. Three different lengths of deletions were observed (Fig. 1). Since none of the deletions changed the reading frame, we suspect that most of these deletions represent viable mutations. Only one late-time-point clone (T69 BL 1-9 in Fig. 1) had a frameshift mutation that was located at amino acid residue 254. None of the other late-time-point clones had in-frame stop codons or frameshifts in gp120 that would be part of defective DNA molecules.

Sequence divergence of SIV variants. The sequence data were used to quantify variation in the gp120 region of *env*. The nucleotide substitution rate was calculated by dividing the total number of nucleotide substitutions by the total number of nucleotide bases sequenced and multiplying by a fraction that accounts for the time of infection. Deletions were not counted as nucleotide substitutions and were subtracted from the total number of nucleotide bases sequenced. For the 10 69-week clones from Mm243-86, there were 152 nucleotide substitutions per 15,686 total bases (1.0%) and 123 amino acid changes per 5,228 total residues (2.4%). The fixation rate of nucleotide substitutions for this group of clones was 7.3×10^{-3} per site per year. For the six 93-week clones from Mm243-86, there were 122 nucleotide substitutions per 9,402 total bases (1.3%) and 102 amino acid changes per 3,134 total residues (3.3%), and the fixation rate of nucleotide substitutions was 7.3×10^{-3} per site per year (Table 1). Nucleotide substitutions thus appeared to accumulate in the virus population over the 24-week time interval between 69 and 93 weeks in Mm243-86 (Table 2).

For the six 43-week clones from the second animal, Mm326-87, there were 90 nucleotide substitutions per 9,444 total bases (1.0%) and 70 amino acid changes per 3,148 total residues (2.2%). The fixation rate of nucleotide substitutions for Mm326-87 was 1.2×10^{-2} per site per year, which is approximately 1.6 times higher than the rate for Mm243-86 (Table 1).

Control experiments. Since the SIVmac239 used to infect Mm243-86 had been propagated in HuT 78 cells for 41 days before inoculation, we performed a control experiment to determine the genetic composition of the infecting virus inoculum. The virus stock used to infect Mm243-86 was inoculated onto fresh HuT 78 cells, and the virus was grown for an additional 31 and 55 days. Analysis of five *env* clones (Fig. 3) revealed only 16 mutations among the 7,875 bases sequenced (0.2%) and only 15 amino acid changes per 2,625 total residues (0.6%) (Table 1). This represents an average of only 3.2 nucleotide substitutions and 3 amino acid changes per envelope. None of the amino acid changes in clones from HuT 78 cells were found in clones obtained from the rhesus monkey. Thus, the vast majority of sequence changes in clones recovered from the two rhesus monkeys accumulated during in vivo infection and not during in vitro cultivation of the virus before inoculation.

To further demonstrate accumulation of changes with time of in vivo infection, five full-length envelope clones were obtained from recovered virus at 6 weeks postinfection from Mm243-86, and the entire gp120 region was sequenced (Fig. 4). Sequence analysis revealed an average of only 6 nucleotide substitutions and 3.8 amino acid changes per envelope. A total of 30 mutations were found among the 7,874 bases sequenced (0.4%), and a total of 19 amino acid changes were found among the 2,624 residues (0.7%) (Table 1). Two of these five early-time-point clones had mutations which would be part of defective molecules; clone T6 V 23-73 had an in-frame stop codon at residue 350, and clone T6 V 23-85 had a frameshift mutation (deletion of one A) at residue 387 (Fig. 4). Four of the nucleotide substitutions found in these early-time-point clones from Mm243-86 were also found in late-time-point clones from the same animal. Although nucleotide substitutions clearly accumulated over the full time course of infection of Mm243-86, the rate of appearance of nucleotide substitutions was higher in the 6-week samples (33 per 1,000 per year) than in the 69- and 93-week samples (7.3 per 1,000 per year) (Tables 1 and 2).

Preferred nucleotide substitutions. Sequence analysis revealed a strong tendency for G→A and A→G transitions, which occurred in 40% (158 of 394) and 30% (120 of 394) of all nucleotide substitutions, respectively, in clones recovered from the two rhesus monkeys. All other nucleotide substitutions were found at frequencies of 10% or less, including the following: 10% T→C, 7% A→C, 5% C→T, 4% T→A, 3% A→T, 1% C→A, 0.5% T→G, and 0.5% G→T.

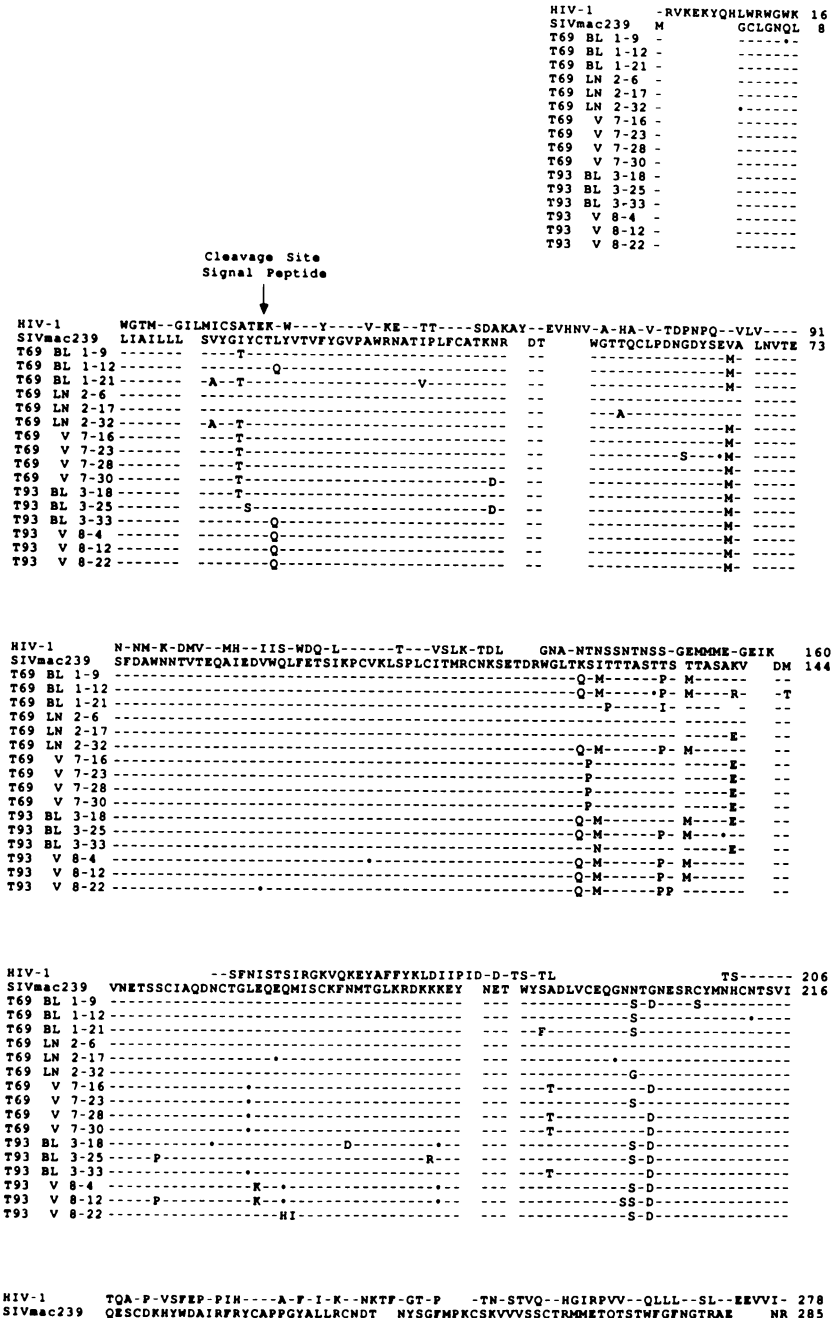

FIG. 1. Deduced amino acid sequences of late-time-point *env* clones obtained at 69 and 93 weeks postinfection from rhesus monkey Mm243-86. T69 and T93 refer to the number of weeks after infection with molecularly cloned SIVmac239. BL refers to blood, LN to lymph node, and V to cultured cells infected with recovered virus as the source of DNA for PCR amplification and cloning. The numbers 1-9, 1-12, etc., are individual clone designations. Shown above the 16 postinfection sequences are the sequences of the SIVmac239 clone (30a) and the HIV-1 lymphadenopathy-associated virus clone (45). Dashes represent amino acid identity; dots represent silent mutations; slashes represent frameshift mutations. Spaces represent gaps introduced to optimize alignment. Brackets labeled C LOOP and CD4 BINDING DOMAIN refer to the V3 cysteine loop which is variable in HIV-1 (40) and the CD4 binding domain of HIV-1 (21), respectively. The signal peptide cleavage site is that of SIV (44). SU, Surface protein (gp120); TM, transmembrane protein (gp41) (22).

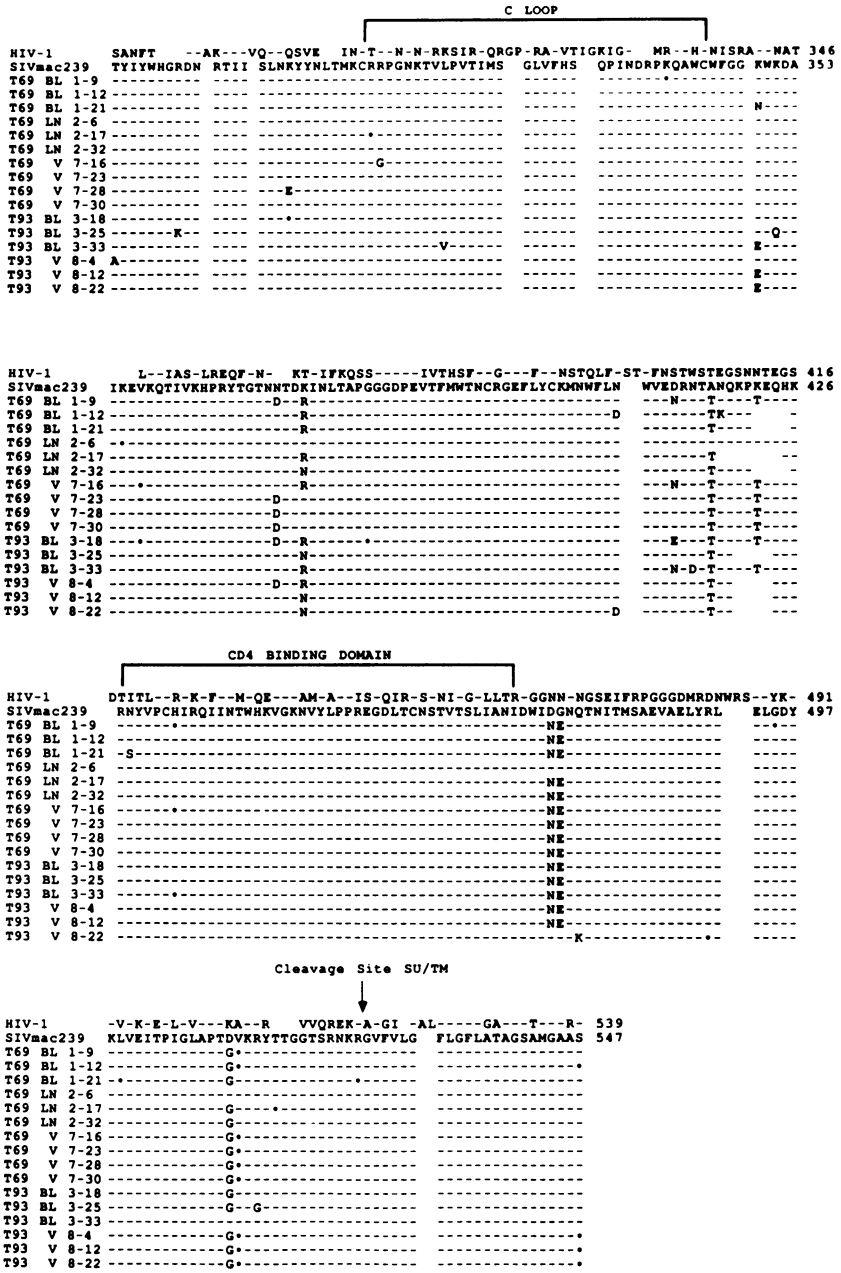

FIG. 1—*Continued.*

Selection for amino acid changes. Analysis of the types of nucleotide substitutions found in *SU* in late-time-point clones recovered from the two rhesus monkeys revealed a strong tendency for nucleotide substitutions that changed the amino acid. Calculation of the proportions of nonsynonymous (amino acid-changing) and synonymous (silent) nucleotide substitutions showed that 81% (295 of 363) were nonsynonymous, whereas only 19% (68 of 363) were synonymous. The average nonsynonymous nucleotide fixation rate (6.8×10^{-3} per site per year) was thus found to be approximately 4.3 times higher than the average synonymous fixation rate (1.6×10^{-3} per site per year).

Within the five variable regions, the pattern of nucleotide substitutions was especially nonrandom. An extremely high percentage of the mutations in these regions changed the amino acid (Table 3). Statistically significant differences were found between the observed frequencies of nonsynonymous substitutions and frequencies of nonsynonymous substitutions expected from random genetic drift. Within the five variable regions of these clones, a remarkable 182 of 186 nucleotide substitutions changed the amino acid.

To further demonstrate the nonrandom nature of sequence changes within the five variable regions, the frequencies of nucleotide substitutions occurring in the first, second, and third codon positions were determined (Table 4). An extremely high percentage of nucleotide substitutions was found in the first and second positions. If mutations had occurred randomly in the variable regions (no in vivo selec-

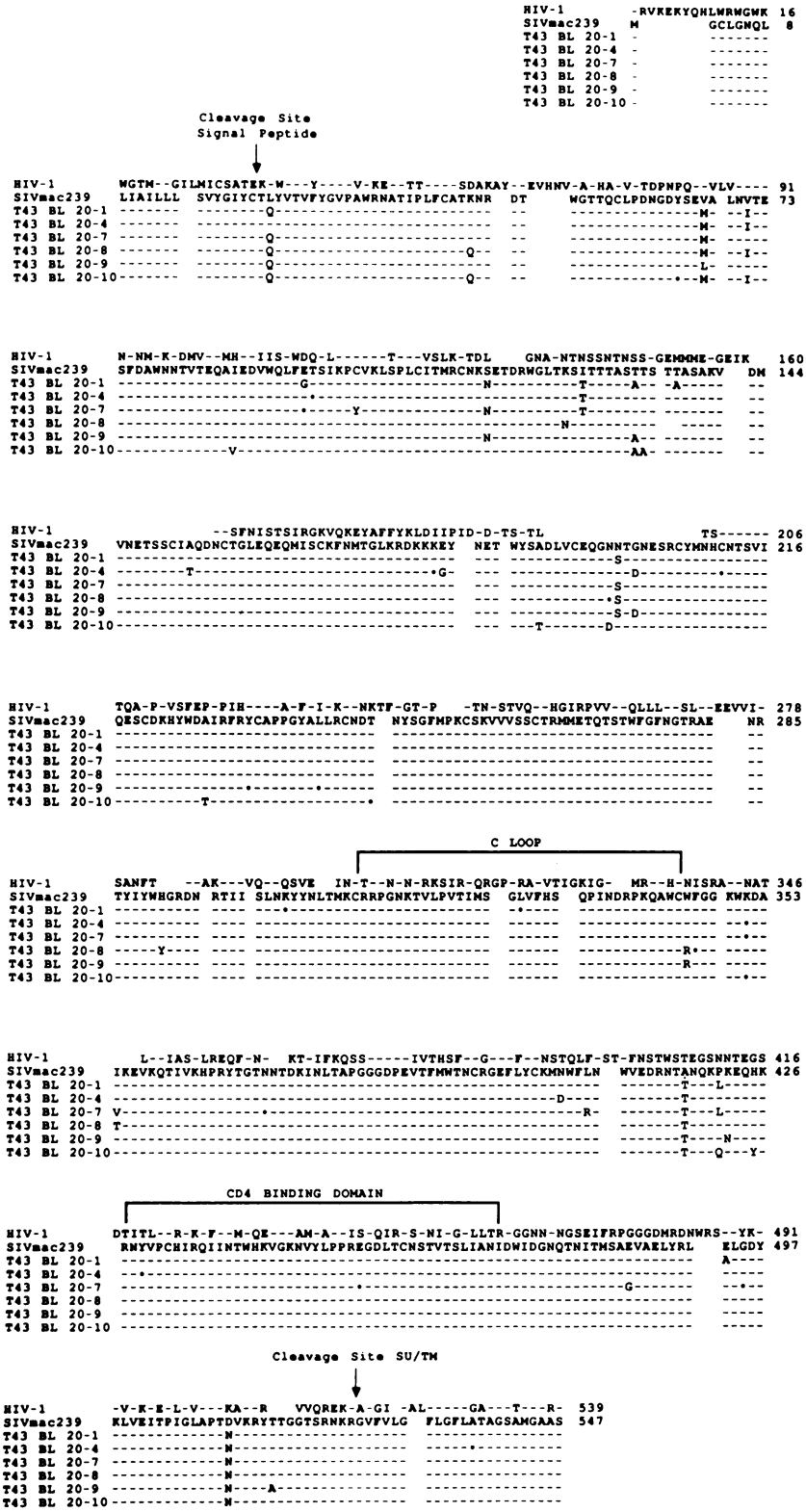

FIG. 2. Deduced amino acid sequences of late-time-point *env* clones obtained at 43 weeks postinfection from rhesus monkey Mm326-87. The layout of this figure is identical to that of Fig. 1. All six sequences were obtained from cloned DNA from a 43-week PBMC sample.

TABLE 1. Summary of sequence changes in gp120[a]

Name of clone	Total no. of:					Fixation rate[b] (nucleotide substitutions per site per year)
	Nucleotide changes	Nucleotide bases	Amino acid changes	Silent changes	Amino acid residues	
93-Week clones, Mm243-86						
T93 BL 3-18	22	1,575	17	5	525	7.8×10^{-3}
T93 BL 3-25	20	1,563	19	1	521	7.2×10^{-3}
T93 BL 3-33	19	1,575	16	3	525	6.8×10^{-3}
T93 V 8-4	22	1,563	17	5	521	7.9×10^{-3}
T93 V 8-12	20	1,563	17	3	521	7.2×10^{-3}
T93 V 8-22	19	1,563	16	3	521	6.8×10^{-3}
69-Week clones, Mm243-86						
T69 BL 1-9	22	1,574	17	5	524	1.1×10^{-2}
T69 BL 1-12	19	1,563	16	3	521	9.2×10^{-3}
T69 BL 1-21	18	1,557	15	3	519	8.7×10^{-3}
T69 LN 2-6	1	1,575	0	1	525	4.8×10^{-4}
T69 LN 2-17	11	1,554	7	4	518	5.3×10^{-3}
T69 LN 2-32	16	1,563	14	1	521	7.7×10^{-3}
T69 V 7-16	19	1,575	15	4	525	9.1×10^{-3}
T69 V 7-23	15	1,575	12	3	525	7.2×10^{-3}
T69 V 7-28	16	1,575	14	2	525	7.7×10^{-3}
T69 V 7-30	15	1,575	13	2	525	7.2×10^{-3}
6-Week clones, Mm243-86						
T6 V 23-72A	5	1,575	2	3	525	2.8×10^{-2}
T6 V 23-72B	4	1,575	3	1	525	2.2×10^{-2}
T6 V 23-73	7	1,575	5	2	525	3.9×10^{-2}
T6 V 23-77	10	1,575	6	4	525	5.5×10^{-2}
T6 V 23-85	4	1,574	3	1	524	2.2×10^{-2}
Control clones, Mm243-86						
CTL72 0-2	2	1,575	2	0	525	6.4×10^{-3}
CTL72 0-6	2	1,575	2	0	525	6.4×10^{-3}
CTL96 00-9	3	1,575	3	0	525	7.2×10^{-3}
CTL96 00-12	6	1,575	5	1	525	1.4×10^{-2}
CTL96 00-16	3	1,575	3	0	525	7.2×10^{-3}
43-Week clones, Mm326-87						
T43 BL 20-1	15	1,575	13	2	525	1.2×10^{-2}
T43 BL 20-4	14	1,575	9	5	525	1.1×10^{-2}
T43 BL 20-7	17	1,575	12	5	525	1.3×10^{-2}
T43 BL 20-8	13	1,569	11	2	523	1.0×10^{-2}
T43 BL 20-9	13	1,575	11	2	525	1.0×10^{-2}
T43 BL 20-10	18	1,575	14	4	525	1.4×10^{-2}

[a] Deletions were not counted in calculations.

[b] Average fixation rates (nucleotide substitutions per site per year) were as follows: for 93-week and 69-week Mm243-86 clones, 7.3×10^{-3}; for 6-week Mm243-86 clones, 3.3×10^{-2}; for control Mm243-86 clones, 8.2×10^{-3}; for 43-week Mm326-87 clones, 1.2×10^{-2}.

tion), then the mutations should have occurred with relatively equal frequencies in each codon position. Statistically significant differences were found between the observed and expected frequencies of mutations in the first, second, and third base positions in four of the five variable regions.

TABLE 2. Accumulation of changes with time of infection in vivo[a]

Time of DNA isolation (wk)	Animal	Avg no. per gp120	
		Nucleotide substitutions	Amino acid changes
6	Mm243-86	6.0	3.8
43	Mm326-87	15.0	11.7
69	Mm243-86	15.2	12.3
93	Mm243-86	20.3	17.0

[a] Deletions were not counted in calculations.

DISCUSSION

One of the key features of SIV- and HIV-induced disease is the nature of the persistent infection. SIV and HIV, like other lentiviruses, have a remarkable ability to persist and eventually induce a chronic, debilitating disease in spite of an apparently strong host immune response to the virus. Infected individuals may remain clinically well for years while maintaining easily detectable humoral and cellular immune responses, only to succumb eventually to the virus. The basic mechanisms by which this persistence and chronic course are achieved are not well understood, but antigenic variation may be one component of the ability of these viruses to avoid elimination by the host immune system.

In this report, we have quantified the extensive sequence variability accumulating in vivo after infection with molecularly cloned virus. We have shown that SIV evolves rapidly in vivo into a complex mixture of genotypes. The rate at which nucleotide substitutions become fixed in vivo is determined not only by the mutation rate per replication cycle and the number of virus replication cycles per unit time but

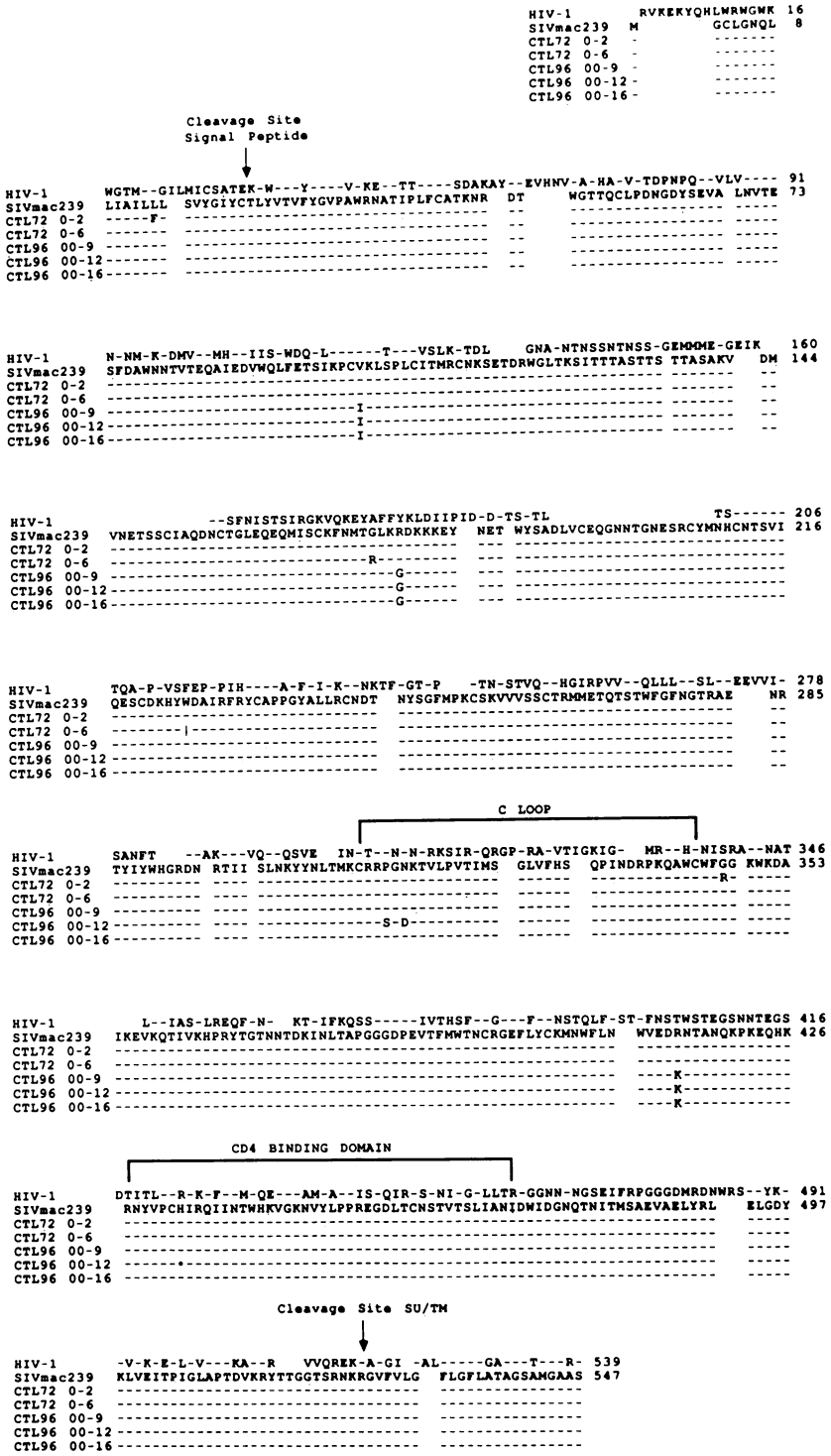

FIG. 3. Deduced amino acid sequences of control clones obtained at 72 and 96 days after infection of HuT 78 cells. The layout of this figure is identical to that of Fig. 1. There is one additional symbol present in this figure; |, stop codon. Sequences CTL72 0-2 and CTL72 0-6 represent clones 72 days after transfection of HuT 78 cells, whereas sequences CTL96 00-9, CTL96 00-12, and CTL96 00-16 represent clones 96 days after transfection of HuT 78 cells. All five sequences were obtained from clones of Hirt supernatant DNA.

also by the fitness for survival of the mutants that appear. The fixation rate of nucleotide substitutions of 8.5 per 1,000 per year in *SU* (weighted average of late-time-point clones from Mm243-86 and Mm326-87) is approximately 10^6 times higher than that of the average mammalian gene (12, 23) and roughly equivalent to rates that have been reported for influenza virus (1, 13) and for type C retroviruses in cell culture (6). Amino acid substitutions were fixed at 2.1 per

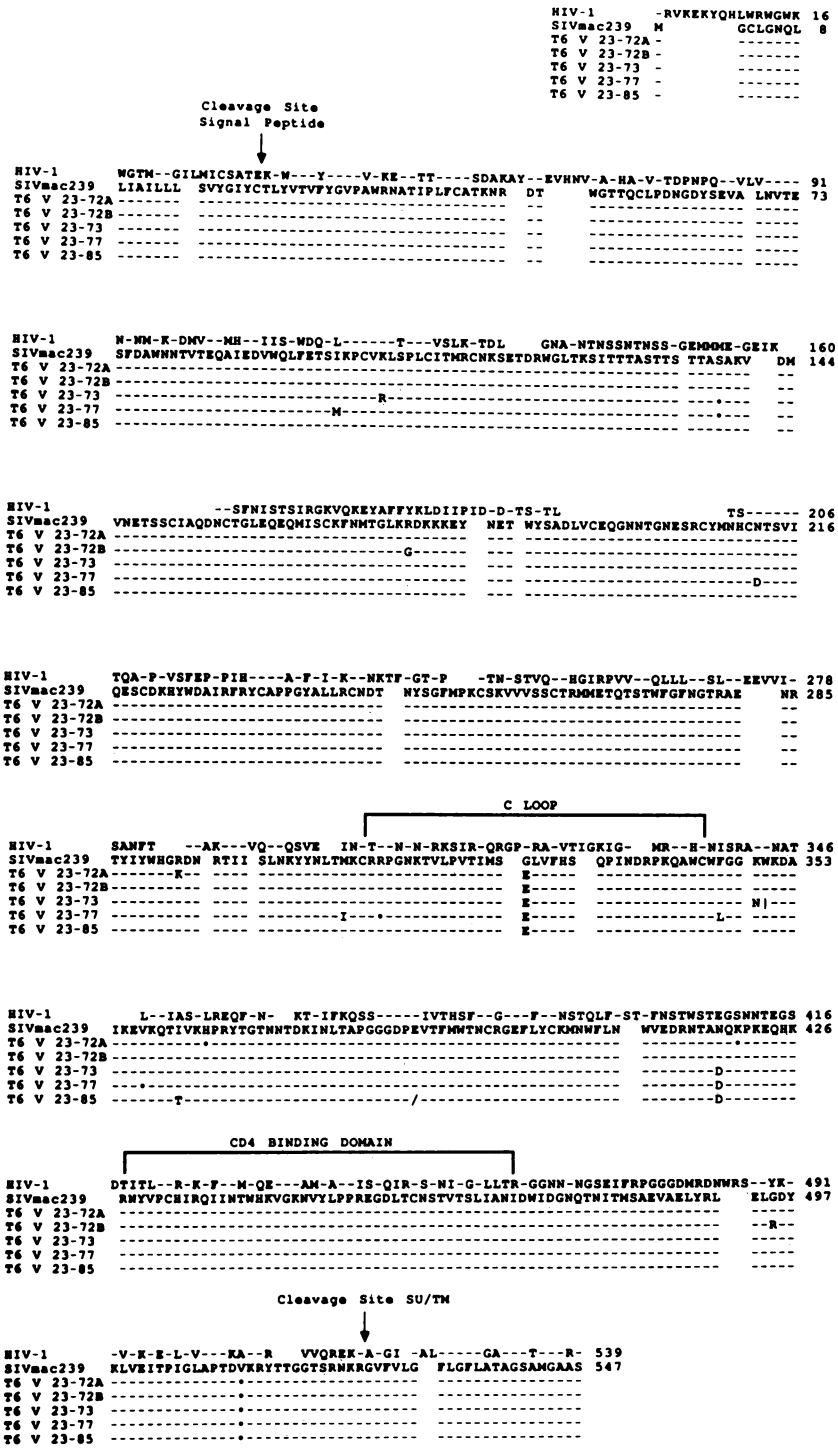

FIG. 4. Deduced amino acid sequences of early-time-point *env* clones obtained at 6 weeks postinfection from rhesus monkey Mm243-86. The layout of this figure is identical to that of Fig. 1. All five sequences were obtained from clones of Hirt supernatant DNA prepared from CEM × 174 cells infected with virus recovered at 6 weeks postinfection.

100 per year. This corresponds to 11 amino acid substitutions per gp120 per year not counting deletions.

The accumulation of changes with time of in vivo infection, the nature of the changes observed, the control experiments, and the clustering of mutations in experimental but not control clones all argue that our analyses of PCR-derived clones provide an accurate reflection, both quantitative and qualitative, of the evolution of sequence changes in vivo. Furthermore, independent PCR amplifications of blood, lymph node, and infected cell DNAs from 69-week samples yielded similar results in terms of the nature and extent of genetic change.

TABLE 3. Selection for amino acid changes in five variable regions in vivo

Variable region	% (no./total) of nucleotide substitutions that changed the amino acid[a]		χ^2
	Observed	Expected (random genetic drift)	
V1	97 (58/60)	73 (44/60)	16.70[b]
V2	94 (34/36)	78 (28/36)	5.79[c]
V3[d]	100 (18/18)	83 (15/18)	3.60[e]
V4	100 (43/43)	84 (36/43)	8.36[b]
V5[d]	100 (29/29)	83 (24/29)	6.04[c]

[a] Late-time-point clones from Mm243-86 and Mm326-87.
[b] Statistically significant, $P < 0.005$.
[c] Statistically significant, $P < 0.025$.
[d] Mm243-86 only.
[e] Statistically significant, $P < 0.100$.

All 27 clones recovered from the two rhesus monkeys were different from each other and differed from the starting clone. This indicates that authentic, independent clones of SIVmac239 were analyzed. Comparison of the 27 sequences also revealed that sequence changes in clones from recovered virus were by and large representative of sequence changes found in clones from PBMC and lymph node tissue taken at the same time. Sequences in variable regions 1 and 4 in clones from recovered virus, however, tended to be more homogeneous than those in clones from blood and lymph nodes, indicating that there may be cell culture selection for populations of virus with specific sequences in certain regions of the envelope gene. Meyerhans et al. (26) have previously presented evidence for cell culture selection for individual *tat* gene variants. Recently, Simmonds et al. (39) observed no cell culture selection for individual *gag* variants.

Although it has been shown for eukaryotic genes that mutation rates among the four nucleotides are not equal (24), the percentage of G→A and A→G transitions in the SIV system described in this report seems extraordinarily high. The high percentage of these transitions might possibly be related to preferential misincorporation by reverse transcriptase. A G→A transition could arise through G:T mispairs formed during first-strand synthesis or through A:C mispairs formed during second-strand synthesis of reverse transcription. Conversely, an A→G transition could arise through A:C mispairs formed during first-strand synthesis or through G:T mispairs formed during second-strand synthesis. High frequencies of such G:T and A:C mispairs have been reported in studies with the HIV-1 reverse transcriptase (30, 31, 46). There may be some structural basis for these mutations in terms of the conformations of particular types of mismatches as seen by reverse transcriptase in copying natural DNA sequences (32). Goodenow et al. (8a) previously reported a strong preference for G → A base substitutions in HIV-1. The deamination of methylated cytosine residues (C→T transition) in integrated SIV molecules is another possible mechanism for the high frequency of G→A transitions (3); however, since methylated C residues in eukaryotic DNA occur almost exclusively at 5′-CG-3′ dinucleotides (5, 9) and since there are only eight such CG dinucleotides within the 1,575 bp of SU, methylation is not a likely explanation. Since the base composition of *SU* is 35% A, 26% T, 22% G, and 17% C, the preponderance of G→A and A→G transitions cannot be explained by a high percentage of G and A in the viral RNA. Finally, misincorporation by Taq polymerase is not a likely explanation for the high percentage of G→A and A→G transitions based on previous experiments on Taq fidelity (43).

TABLE 4. Nonrandom nucleotide substitutions

Variable region	% (no./total) of nucleotide substitutions that occurred in the indicated position[a]				χ^2
	Observed			Expected (random nucleotide substitutions)	
	First base	Second base	Third base		
V1	54 (33/61)	26 (16/61)	20 (12/61)	33 (20/61)	12.24[b]
V2	19 (7/37)	76 (28/37)	5 (2/37)	33 (12/37)	31.74[b]
V3[c]	33 (6/18)	44 (8/18)	22 (4/18)	33 (6/18)	1.33
V4	66 (29/44)	25 (11/44)	9 (4/44)	33 (15/44)	22.21[b]
V5[c]	52 (15/29)	48 (14/29)	0 (0/29)	33 (10/29)	14.10[b]

[a] Late-time-point clones from Mm243-86 and Mm326-87.
[b] Statistically significant, $P < 0.005$.
[c] Mm243-86 only.

In our study of SIV *env* variation, we have observed not only a high percentage of G→A and A→G transitions but also a statistically significant proportion of nucleotide substitutions occurring in the first and second codon positions (Table 4). The observed pattern of nucleotide substitutions might therefore be related to selection of preferred amino acid substitutions in the SIV envelope glycoprotein. A full 81% of the nucleotide substitutions in *SU* in late-time-point clones from two rhesus monkeys changed the amino acid. The fixation rate of nonsynonymous nucleotide substitutions (6.8×10^{-3} per site per year) was found to be approximately 4.3 times higher than the fixation rate of synonymous mutations (1.6×10^{-3} per site per year). Within the five variable regions, a remarkable 98% (182 of 186) of the nucleotide substitutions changed the amino acid, and statistically significant differences between observed frequencies and frequencies expected from random genetic drift were found (Table 3). The difference in nonsynonymous versus synonymous fixation rates in the SIVmac *env* gene contrasts markedly with rates for eukaryotic genes. Li et al. (25), for example, have shown that the nonsynonymous mutation rate for eukaryotic genes is only one-fifth the synonymous mutation rate. Quite clearly, this usual predominance of synonymous mutations results from severe limitations on the numbers of amino acid changes that will still allow optimal protein function. Like cellular genes, the HIV-1 *gag* gene shows a bias toward synonymous mutations (39). These observations, together with the lack of focal accumulation of substitutions in the variable regions of *SU* in virus passaged for 3 months in cell culture, suggest that there are strong selective forces for amino acid changes in certain segments of the SIV envelope gene in vivo.

Five variable regions have been identified in late-time-point clones from Mm243-86 and Mm326-87. Four of these five variable regions of SIV (V1, V2, V4, and V5) correspond to variable regions V1, V2, V4, and V5 of HIV-1 (40) (Fig. 5). Variable regions 1 and 2 of SIV do not correspond to any functional domains or epitopes identified in the HIV-1 system. Variable region 3 of SIV is located in a region which corresponds to a weak cytotoxic T-lymphocyte epitope of HIV-1 (41). Variable region 4 of SIV, located immediately upstream of the region corresponding to the HIV-1 CD4 binding domain (21), does not correspond to any known functional domains or epitopes in the HIV-1 envelope. Finally, variable region 5 of SIV gp120, located immediately downstream of the region corresponding to the HIV-1 CD4

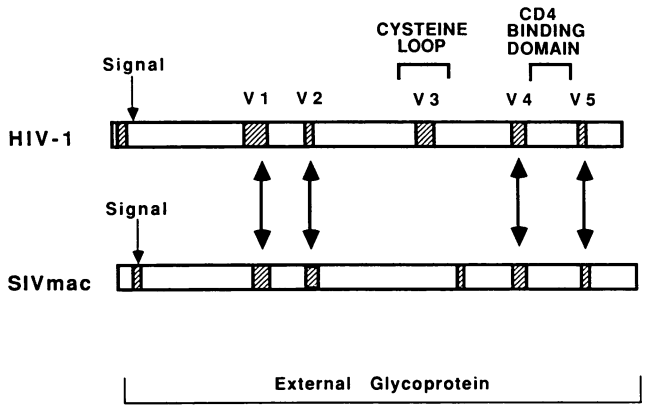

FIG. 5. Summary of variable regions of HIV-1 and SIVmac in *SU*. Variable regions V1, V2, V4, and V5 of SIVmac correspond to variable regions V1, V2, V4, and V5, respectively, of HIV-1.

binding domain, corresponds to a previously reported neutralization epitope of HIV-1 (15). The V5 region of SIV is the only variable region that corresponds to any of the HIV-1 neutralization epitopes identified to date.

One surprising result from our study was that the region corresponding to the variable V3 cysteine loop of HIV-1 was not found to be variable in SIVmac239; this region was found to be quite conserved among the 27 clones analyzed. The variable V3 cysteine loop of HIV-1 is believed to be a major target for neutralizing antibodies (8b, 33). RP135, a 24-amino-acid peptide from this region (located between amino acid residues 300 and 337 in Fig. 1), blocked fusion inhibition of antisera raised against the entire HIV-1 *env* or against the central region of *env* and also blocked the activity of serum from a chimpanzee infected with HIV-1 III_B (33). It will be interesting to see whether this region is a major neutralization epitope of SIV, as it is in HIV-1. In addition to being a neutralizing determinant, the V3 cysteine loop of HIV-1 has also been reported to contain a major cytotoxic T-lymphocyte epitope (41).

The selective pressures that contribute to the pattern of variation have not been defined. However, the host immune response is likely to be one important driving force. Whether the SIV variable regions identified in this study are targets of humoral or cell-mediated immunity and whether sequence variation in these regions allows the virus to evade ongoing antibody and T-cell responses are important questions for future investigation. Recently, Haigwood et al. (11) presented strong evidence for the role of at least three HIV-1 gp120 variable regions in recognition by neutralizing antibodies. Variable regions may influence neutralizing antibody recognition in direct ways, by serving as target epitopes (33), or in indirect ways, by changing three-dimensional or conformational epitopes from a distance (17, 29, 47). The system and reagents defined in this report will allow us to investigate in exquisite detail the significance of sequence variation for recognition by neutralizing antibodies. Sera from these animals have been stored at numerous time points after infection, and the variant envelope genes were cloned in such a way as to allow easy replacement into cloned virus.

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